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ALTERATIONS IN CELLULAR
AND METABOLIC PROCESSES

FINAL REPORT

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ALTERATIONS IN CELLULAR AND METABOLIC PROCESSES

The cellular morphology, intracellular structure and biochemical characteristics of a cell species can be used in numerous ways to elucidate the functional aspects of that cell, or a cell population. Most cell species have several specific biochemical activities which may be unique in respect to their contribution to the maintenance of the normal physiologic functions of the organism.

Alteration in cellular morphology, intracellular structure and biochemical activities may occur under exposure to adverse conditions as the result of infection, onset of disease or as a result of many other considerations. At the same time, the recognition of abnormal cell morphologies or altered biochemical characteristics of a cell species, may signal the onset of disease, a response to an infection or other abnormal conditions which would affect the normal status of a cell.

With the research tools and biochemical methods used to study cells or their biochemical products and constituents, scientists have been devising methods of analyzing smaller and smaller entities of the single cell, or to perform assays on a tiny sample with an even lower range of detectability than the year before.

The primary objective of the research performed under this Grant was to implement the use of specialized instrumentation (ie. the Universal Microspectrophotometer (UMSP-I) manufactured by Carl

Zeiss, Oberckoken, Germany and a PDP-12 computer manufactured by Digital Equipment Corporation, Maynard Mass.) for analyzing single cells to obtain specific biochemical and morphological information. Rapid-scanning microspectrophotometry and computer-assisted data analysis can provide specific information about a cell or a cell population, which can not be determined by other means. Furthermore, data obtained from as few as 25-100 cells can supply sufficient information to arrive at specific conclusions pertaining to a particular cell population in regard to certain biochemical determinations.

This work was performed as an initial portion of the Early Detection of Disease Program (EDPRO) in the Cellular Analytical Laboratory of the Life Sciences Directorate at the Johnson Spacecraft Center, Houston, Texas. Specific projects were undertaken to establish and verify procedures and to establish certain parameters of cellular biochemical and metabolic functions at the single cell level, in order to apply these techniques to specific projects related to spaceflight mission experiments.

Microspectrophotometric Analysis of the Cell Cycle

The application of rapid-scanning microspectrophotometry and computer-assisted data analysis of normal and neoplastic cells and cell transformations in neoplasias have been described by Wied et al. (31,32). Cytophotometric studies without automated data collection and analysis have been reported for a variety of cell species (1, 8, 10, 11, 12, 13, 15, and 20). More recent studies have been directed toward quantitative determinations of DNA and RNA in cells stimulated with phytohemagglutinin or other similar non-specific stimuli (16-18 and 24) and Bartels et al. (2) have studied stimulated lymphocytes by cytophotometry and computer analysis.

In an attempt to assess the physiologic and certain probable immunologic properties of various peripheral leucocytes, it was necessary to define the quantitative biochemical aspects of these cells during the cell cycle. Since there appears to be a proliferation of lymphocytes during the immune response, which may later lead to the synthesis of globulins and specific antibody, the intracellular characteristics would be quite different at any stage of the cell cycle while the gross morphologic appearance would not necessarily be different from that of a normal, unstimulated cell.

To achieve characterization of these cytophotometric aspects of the lymphocyte, a rapidly proliferating lymphosarcoma was used

as a source of lymphoid cells undergoing rapid cell division.

Materials and Methods:

Lymphocytes were obtained by removing a transplanted lymphosarcoma from a DBA/1J mouse. The tumor line was maintained by serial transplant. A small peripheral portion of the tumor was dissected and minced with scissors in 0.89 per cent sodium chloride. Following mincing, the cells and tissue pieces were separated by low-speed centrifugation for 30 seconds. The supernatant containing primarily single cells was used as the sample. The cells were washed once in fresh saline and then a hematocrit tube was used to apply a thin layer of cells to a quartz slide which was allowed to air-dry. Following air-drying the cells were fixed for 10 min in absolute ethanol. The fixed specimen was again air-dried and could then either be immersed in glycerin to which a protective quartz cover glass was added and sealed in place with clear lacquer or the specimen could be stored air-dried until ready for examination.

Cytophotometric analyses of the cells were performed using a Zeiss Universal Microspectrophotometer (UMSP-I) with simultaneous data collection using a Facit high-speed tape punch. The cells were analyzed by scanning 0.5 micron spots at 0.5 micron intervals and at 260 nm.

Data analysis was initiated by entering the raw data into the high-speed reader of a PDP-12 computer and subsequent storage on LINC tapes. Editing, data reduction and initial statistical

analyses were accomplished by using various TICAS routines (31). Figure 1 shows a generalized scheme for cytophotometric analysis using the UMSP-I and the PDP-12 computer. A complete description of the instrumentation operation and specifications has been described by Wied et al. (31 and 32).

Results and Discussion:

Microscopic examination of the specimen revealed that approximately 90 per cent of the cells were of a rather uniform size, while 3-5 per cent were obviously larger and the remaining 5-7 per cent were recognized as mitotic figures. This observation was in general agreement with the established distribution of the stages of the cell cycle (14).

Post-mitotic daughter cells nearing cytoplasmic separation appeared to be the same size as the majority of the cells. This size correlation brought forth the assumption that the large percentage of the small cells of the population should be identified as G-1 phase (juvenile or presynthetic). The slightly larger cells of this population should therefore be in the S phase, with less than 10 per cent of the population positively identified by visual observation as pre-mitotic (G-2) or mitotic (M) cells.

Microspectrophotometric analysis of the smallest cells produced raw data matrices such as that shown in Fig. 4. As the size of the cell increased, the corresponding raw data matrix exhibited density values which also increased (Figs. 5-7). The cell from

which the raw data matrix of Fig. 8 was obtained was visually recognized as prophase (early G-2) by the degree of chromatin condensation of the DNA. In Fig. 9, the matrix of a typical metaphase (M) cell is shown and Fig. 10 shows the matrix for a mitotic cell. Figure 11 was similar to Fig. 10, however it was evident that chromatin decondensation had occurred and cytoplasmic separation was nearly complete.

The effect of chromatin coiling and condensation relative to their contribution to density can be seen by comparing Fig. 8 with Figs. 9 and 10. When the summation of optical density (OD) values in Fig. 4 (G-1 phase) was compared with that of Fig. 8 (G-2 phase), there is almost an exact doubling of the nucleoprotein which is representative of the 2C nuclear content.

In Fig. 11, where the daughter cells exhibit decondensation, each juvenile cell is basically identical to the cells recognized as those comprising the G-1 population. An important feature which was demonstrated in the data of this rather homogeneous population of cells was that the size differences observed were more directly related to biochemical events of the cell cycle than heterogeneity of the cell size. At the same time it also emphasizes the fact that variation in peripheral blood lymphocytes may be directly related to cell origin and biochemical function.

In Fig. 12, a summary of microspectrophotometric data of a series of cells which was representative of the complete cell

is shown. The cells were from a preparation which was alcohol fixed and unstained. All the examples were scanned at 260 nm. Another series of cells which had been stained by the standard Papanicolaou method were scanned at 530 nm. The data from this series is shown in Figs. 13 and 14 and the profiles of the series were similar to those from the unstained cells.

As a result of the data collected from these series, and when considering the time factors related to the events of the cell cycle, it is in most instances, a very straight-forward procedure to determine the basic LC content of a cell population. If biochemical or cellular differences are suspected in a given population, the application of microspectrophotometry and associated computer analyses can provide useful results. This type of analysis can also be of special significance where only a small number of cells may be available for examination.

Cytophotometry of Virus-Infected Tissue Culture Cells

Polioviruses were the first viruses shown to cause cytopathic changes in tissue culture cells and thereby serve as an indicator of viral replication (23). Animal cell cultures are widely used to grow viruses for diagnostic purposes and the detection of virus in the culture is of primary significance in many types of laboratory procedures.

In a number of virus infections, the time-course and type of cellular alterations are used as criteria in viral identification. Some of the common morphological features exhibited by infected cells are development of nuclear or cytoplasmic inclusion bodies, vacuolation, rounding of cells, giant multinucleated cells, stranding and irregular proliferation features. In many instances, upon the appearance of one or more of these morphological features in the cell culture, a tentative identification of the virus group is possible.

Viruses such as influenza or rubella replicate in cell cultures without producing any visible cytopathic alterations. In these types of viral infections, techniques such as hemadsorption, interference or fluorescent antibody techniques are used to determine the presence of non-cytopathic viruses. Greater detail can be seen in fixed cultures stained with Giemsa or hematoxylin and eosin, or by the use of cytochemical staining techniques which are specific for nucleic acids.

Rapid-scanning microspectrophotometry and associated computer analysis of data has been successfully employed in distinguishing between morphologically identical normal embryonic lung and epidermoid carcinoma tissue culture cell lines (31 and 32) which could not be reliably distinguished by standard cytological and cytochemical methods. From the conclusions reached in these studies concerning the discriminating capacity based on computer analysis of microspectrophotometric data, it is evident that these procedures have direct application to the determination of virus in tissue culture cells. These techniques would be of particular interest in determining the presence of non-cytopathic viruses and it would be of even greater significance if virus-infected cells could be identified at an earlier time in the early infective stage than what is now possible using standard procedures.

Materials and Methods:

The cells used for this study were WI-38 tissue culture cells grown in Hank's Basal Medium. Coxsackie B-5 virus was used as the infective agent. This virus was used because of the well established and easily recognized cytopathic effects on culture cells. Specimens for microspectrophotometric analysis were prepared when it was visually estimated that 45-50 per cent and later when 95 per cent of the culture cells exhibited cytopathic alterations. Control cells were prepared with each experimental sample.

The cultures were treated with versene to release the cells from the culture tubes. This also caused separation of the cells from each other. The specimen was washed gently in 0.85 per cent sodium chloride twice and a small aliquot of the cells layered onto a quartz slide. Following air-drying, the specimen was fixed in absolute ethanol and air-dried again prior to mounting for analysis. The instrumentation and procedures used for cytophotometric analysis have been described elsewhere (31 and 32).

For each specimen, a population of 50 cells was examined. The data was collected on paper tape and later entered onto LINC tape on a PDP-12 computer for editing and data analyses.

Results and Discussion:

Population histograms of the frequency distribution of OD values of control and infected cells are shown in Figs. 15 and 16. Even though the control cells were prepared at each of the sampling times, there was remarkable correlation between the two population histograms indicating the stability and uniformity of the cell line. In both the 45-50 and the 95 per cent infected cells, there is a distinct shift in their respective population histograms from that of the controls.

The degree of population infectivity based on cytopathic cellular alterations was assessed by use of Equiprobable Distribution Profile Analysis (3) of individual OD values of each cell. To obtain composites of controls for purposes of comparison

with cells in the infected cultures, both control populations were grouped and a population histogram of all OD values (0.01-1.75) was obtained. The low 25 per cent of the values was determined as any OD value between 0.01 and 0.25 (both values inclusive), while the high 25 per cent of the values was represented by 0.51-1.75 (both values inclusive). These values were then used as descriptors for obtaining pattern distribution profiles of individual cells in the control population.

Figures 17 and 18 show how the data was cleaned and edited to eliminate any undesirable data values and how raw data could be handled in regard to processing for analysis by equiprobable distribution profiles. In Fig. 19, the maximum and minimum profiles for controls is compared with that of infected cells. The (o) represents the location of a low value and the (•) indicates the location of a high value. In all cells of the control population a thin peripheral pattern and a crescent shaped central pattern was present. The control descriptors were then imposed upon the cell matrix of individual cells from the infected populations. Patterns similar to those of the controls were exhibited by some cells (these cells were designated as non-infected), while distinctly different patterns were exhibited by other cells of the populations (these cells were designated as infected).

This type of analysis procedure showed there was extremely

close correlation between the number of cells in each population identified as infected (51 and 96 per cent respectively) and the original estimation of infectivity (45-50 and 95 per cent) which was arrived at by visual estimation of cytopathic alterations exhibited by the infected cultures.

On the basis of the data established from this study, it is possible that similar results could be obtained by analysis of African Green Monkey kidney cells which were infected with rubella virus. Since rubella is a non-cytopathic virus, the time-course for infection could vary from 7-10 days. Infection could be verified by challenge with Echo 11 virus. The interesting aspect of this study could be in arriving at a determination of infection of the cells based on microspectrophotometric data analysis after 4-6 days following inoculation. If this determination were possible, then a number of avenues of research and application utilizing rapid-scanning microspectrophotometry and computer-assisted data analyses could be pursued.

Rapid Scanning Microspectrophotometry of Colorless Euglena gracilis and Astasia longa. A Basis for Differentiation*

Rapid scanning microspectrophotometric studies were undertaken on colorless Euglena gracilis var. bacillaris and Astasia longa to show that the 2 organisms could be reliably distinguished from each other, even though their cellular and structural characteristics are quite similar. With the development of ultraviolet microspectrophotometry by Caspersson et al. (7), new approaches to cytologic and cytochemical studies of tissues and cells were made possible, particularly in quantitative determinations of nuclear DNA and RNA. Further impetus in the application of this type of instrumentation to cytology has been contributed by Wied et al. (31-32) and Bartels et al. (3), in the development and application of computer programs for data analysis and automated cell recognition.

The purpose of this study was to determine whether techniques such as those employed by Wied et al. (31) to differentiate 2 structurally identical tissue-culture cell lines (one normal and one carcinomatous) could be applied to differentiating 2 structurally similar colorless euglenoids. In this report the distinction between the 2 organisms was based on quantitative differences of ultraviolet absorption values at 260 mμ of the individual cells.

The work described in this section was originally published in The J. Protozool. 190:150-155, 1972.

The primary distinction between cells was made by computer-assisted separation according to quantitative differences of nucleoprotein of cell nuclei.

Materials and Methods:

Cultures of Euglena gracilis var. bacillaris (colorless) Pringsheim and Astasia longa Gross were obtained from the Indiana Culture Collection in Polytomella medium (28). Subcultures were prepared in Polytomella medium and were maintained at room temperature (25-27 C) with continuous lighting.

Specimens for examination were prepared immediately upon receipt of the organisms as well as from log-phase cultures sampled at later dates. Examination specimens were prepared by removing 2 ml of culture medium containing the organisms and centrifuging at a low speed in a table-model centrifuge for 1 min. The culture medium was removed and the organisms were resuspended and washed for 1 min in 0.135 M phosphate buffer (pH 7.0). The cells were again sedimented and then resuspended in 0.05 ml phosphate buffer. A hematocrit tube was used to dispense the cell concentrate on a quartz slide. (Quartz slides and coverglasses, manufactured by Carl Zeiss, Inc., Oberkochen, Germany, were made especially for use on the Universal Microspectrophotometer). Upon air-drying, the specimen slides were immersed in absolute ethanol for 10 min, after which they were removed and again air-dried. Quartz coverglasses carrying a drop of immersion glycerol (nd-1.455 at 20 C) were placed on

the specimens and sealed in place with clear lacquer.

Specimens were examined with a Universal Microspectrophotometer (UMSP-I) manufactured by Carl Zeiss, Inc., and equipped with a rapid-scanning motor attached to the microscope stage assembly. Light intensity of the system was measured by a photo-cell and transmitted to an analog-to-digital converter which was coupled to a Facit high-speed paper tape punch for recording the cell scan data. Individual cells were scanned at 260 mμ with a 1 μ spot at 1 μ intervals on both the X and Y axis throughout the cell. The cell scan data which was recorded on the paper tape was fed into the high speed reader of a PDP-12 computer (Digital Equipment Corporation, Maynard, Mass.) and submitted to analysis by TICAS (Taxonomic Intra-Cellular Computer Analytic Systems) programs (3, 31 and 32). Detailed descriptions of the instrumentation as well as comprehensive discussions of computer analysis programs have been given elsewhere(3, 31 and 32).

Results:

Wet mounts of the cultures were prepared and examined by light microscopy to determine that normal structural characteristics were present in the organisms before and after centrifugation and washing. As a result of air-drying the organisms on the specimen slides, and because of their highly plastic nature, the structure of fixed cells ranged from typically elongated to spherical. In this study, spectrophotometric measurements were

performed on random-picked cells without respect to structure or size.

An example of a digital print-out of the OD values (raw data matrix of an Euglena cell scanned at 260 mμ, showing the relative distribution pattern of RNA and DNA) is shown in Fig. 20. In this study a total of 223 organisms (110 of A. longa and 113 of E. gracilis) were examined on the UMSP-I and classified by computer analyses of the cell scan data.

A histogram of total OD (the summation of all the numbers forming the digital image of a cell) for each organism of both populations is shown in Fig. 21. Although there is a difference in the means of the 2 populations, the overlap is too great to use the total OD value of a cell as an identification descriptor with any degree of reliability. Because of the variation in RNA and DNA content, individual cells within each population had a wide distribution of total OD values (Fig. 21). On this basis, an Euglena cell with a low RNA and DNA content would be grouped with an Astasia cell with a high RNA and DNA content, while only Astasia cells with low total OD values (low RNA and DNA content) were distinctly separated from Euglena cells having high total OD values (high RNA and DNA content). These groupings or separations were directly related to the stages of the cell cycle. It should be noted that if the 2 species had been characterized by the same, or nearly the same, DNA content, the above grouping and separations would not have been observed.

When the raw data print-out images of cells from both populations were compared, it was found that nuclear OD values were distinctly different. Most of the OD values for the nucleus of Euglena cells were usually 40 or more, while the OD values for the nucleus of Astasia were 30 or less. It was also noted in some cells that the nuclear OD values were almost identical, yet the total OD of the cells could be highly different because of the cell size and RNA content.

At this point the Pattern Search program of TICAS 12 (31) was selected and various specific patterns were entered into the sub-routine for comparison of nuclear OD values in the 2 populations. Examination of individual cell histograms of selected cells from both populations which had similar histograms provided an estimate of the parameters to be inserted into the sub-routine. After a series of population comparisons using various patterns and OD ranges, it was found that the highest degree of separation of Euglena from Astasia was achieved using the following pattern:

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  3 3
  3 0 0 3   3 = any number between 30-125
  3 0 0 3   0 = any number
  3 3

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The results in terms of "fits" or "matches" of this pattern to the nucleus of each Euglena and Astasia is shown in Fig. 22. Figure 23 is an example of a digital matrix of an Euglena having a nuclear fit of 4 (i.e., when the above pattern with its specific OD range was compared with the digital matrix of the cell, 4

different "matches" or "Fits" occurred over the nucleus). By setting the limits for Astasia as 0 or 1 fit, and Euglena as 2 or more fits, the lowest percentage of misclassifications for each population was obtained.

After examination of the raw data matrix of cells (ie., Fig. 20), it was noted that most of the cells of both groups generally had several random points within the cell-scan boundary in which unusually high OD values were recorded, while within the same cell, but at other scattered points, an unusually low value or no OD value was recorded. The reasons for these phenomena can be reasonably accounted for by consideration of observations made during an electron microscope study (26). One difference noted during the electron microscope study was that the thin sections of the colorless Euglena generally contained a greater number of phospholipid vesicles than did the thin sections of Astasia. Another significant difference was that electron-dense "caps" partially surrounded some of the paramylum grains in colorless Euglena, while such "caps" were absent in Astasia (26). In view of these observations, and since both the phospholipid vesicles and the electron-dense "caps" associated with some of the paramylum grains are proteinaceous cellular constituents, it is believed these structures may have contributed to light scattering of the measuring beam which resulted in the high OD values (excluding nuclear OD values) in the Euglena cells.

In reference to the low OD values or points in the cell-scan

where no OD was recorded, it was believed this was caused by a focusing and light-refraction phenomenon of paramylum grains being directly positioned in the measuring beam light path, and below the focal plane. Depending on the degree of light enhancement caused by a paramylum grain in the measuring beam path, the actual value of the absorption was lowered or completely negated. By locating the bright spot in the cell in the light path of the measuring beam and then changing the focal plane, the bright spot diminished and a paramylum grain was brought clearly into focus. In the case of the zero OD values, this gave the digital matrix of the cell an appearance of having "holes" in it. Furthermore, the percentage of zero values per cell was greater in the Astasia population than in the Euglena population. The difference in the occurrence of the zero value in the 2 populations was also in direct correlation with an observation made during this study of a greater abundance of paramylum grains in Astasia cells than in Euglena, as well as with the same observation made during an electron microscope study of the organisms (26).

Another feature which was observed during this study, but which was not used as a basis for distinction between species was the appearance of the pellicle of the fixed cells when viewed with ultraviolet light. In Astasia, most of the cells had an uneven pellicle outline, appearing as though the pellicle had stretched over the paramylum grains, while in colorless Euglena, the pellicle outline always appeared very smooth.

Blum et al. (6) compared various cellular aspects of the 2 organisms with the electron microscope. The only major distinction between the pellicle of the 2 organisms was that in cross section Astasia was characterized by 36 pellicular ridges, while Euglena had 40 ridges (6). Other than this, distinct structural differences of the pellicle have not been reported.

Discussion:

Leedale (19) reviewed the criteria on which the taxonomy of euglenoids is based. In some of the organisms of this group, as well as in other taxa, distinction between 2 members in the classification scheme may be largely based on structure. If the cells being examined do not have the typical structure, identification becomes questionable or impossible. In this study, cell structure was disregarded as a descriptor, while nuclear absorption values of the individual cells, to which a specific pattern was fitted, have been shown to be a reliable means of distinguishing between the 2 species.

The primary objective of this investigation was to attempt to differentiate between 2 structurally similar organisms on the basis of computer-assisted analyses (3, 31-32) of data obtained by ultraviolet rapid-scanning microspectrophotometry. It was also the original intent of this study to disregard cell structure, because of the plastic nature of the organisms, and to show that the varied structures of the fixed cells of Astasia and Euglena

would not affect cell identification. After examination of only a few organisms in each group, it was evident that significant results could be obtained by using quantitative differences of nucleoprotein in the cell nuclei of the 2 species as a descriptor.

Since the OD values of the cell nucleus were used as the basis for distinction between the 2 organisms, the number of misclassifications in each population (7 of Euglena identifies as Astasia and 8 of Astasia identified as Euglena) does not seem unreasonable when considering that asynchronous, log-phase cultures in which cells in all stages of the cell cycle were present were used for specimen preparation. Upon relating this to the time factor for each stage of the cell cycle, the number of misclassified cells in each population can reasonably be accounted for in the following manner: If a nucleus of an Astasia cell contained the full complement of duplicated DNA prior to nuclear division, then this is the type of nucleus, as viewed in relation to the overall population, that could result in a misclassification as Euglena. At the same time, some Euglena cells were misclassified as Astasia because of their low nuclear absorption values, which indicated they were young daughter cells and their nuclear pattern was on the low end of the nucleoprotein distribution profile for the Euglena population.

Upon examination of the print-out matrix of the Euglena cells which were misclassified as Astasia because of low OD values for the nucleus, it was noticed that if the value of only

one or 2 points within the nuclear area had been 30 or higher, rather than 29 or less, a nuclear pattern fit of a least 2 would have been achieved in 4 of 7 misclassified cells.

Comparison of nuclear values of the Astasia cells misclassified as Euglena with the average cell of the Astasia population showed that the nuclear values were essentially double that of the average Astasia nucleus. By correlation of these data with those from a study on cell division in a mouse lymphosarcoma cell line (25) further evidence in support of the reasons for misclassification was obtained.

An advantage of the organisms used in this study was that the quantitative differences of nucleoprotein of cell nuclei provided a distinct descriptor for cell recognition. Had the nucleoprotein content been the same in the 2 species, then other descriptors would have been used to achieve separation in much the same manner as Wied et al. (31) used to differentiate between normal and carcinoma tissue culture cells which were structurally similar.

Leedale (19) reported a chromosome number of 45 for E. gracilis and estimated that there are 18 in Astasia klebsii. Although we cannot find documentation of a chromosome count for bleached E. gracilis, there is no reason to believe that the chromosome number is changed. Since there is an estimate of 18 chromosomes for A. klebsii (19), it is possible that A. longa would also have about 18 chromosomes. Based on this assumption,

There should be a quantitative difference between the nucleoprotein content of A. longa and E. gracilis. Although no attempt was made to determine the number of chromosomes in A. longa by standard techniques, this study has shown there is a quantitative difference in the relative amount of nucleoprotein of nuclei in the 2 species. By comparison of data on the relative amounts of nucleoprotein in E. gracilis and A. longa, and considering that colorless Euglena has 45 chromosomes, it is suggested that A. longa would have about half as many chromosomes as E. gracilis, which is well within reason regarding the original assumption that A. longa might have approximately 18 chromosomes.

It should also be noted that if these 2 organisms had been characterized by having quantitatively similar nuclear values resulting from similar nucleoprotein content (ie., both organisms having the same chromosome number), then other methods and descriptors for computer analyses would have been employed in an attempt to distinguish between the species.

In view of the difficulties which one encounters in classification schemes, and where there are size and structural variations in the organisms in question, it is evident that methods such as those used in this study to achieve distinction between highly similar organisms are not only feasible, but practical. Even though many structural forms were present in the fixed cells examined in this study, the grouping or separation of cells was dependent upon cellular features (ie., quantitative determina-

tion of nucleoprotein, etc.) other than size or shape.

This is not to say the structure need not be considered, because there are numerous instances in which cell structure could be used as a criterion for characterization and identification, especially where cells with fixed but highly similar structures are being compared or differentiated.

It is also apparent that methods such as those described in this report to distinguish between colorless Euglena and Astasia can be applied to other situations where the similar structures of 2 organisms might lead to questionable or mistaken identification. It would also be possible to employ these techniques to characterize or identify organisms with an unusual structure resulting from cultural or environmental conditions. These methods would also have direct application in identification and characterization of sub-species or varieties as well as mutants.

As a result of this study it is possible to foresee numerous applications of this type of instrumentation and method of data analysis to a wide spectrum of biologic, physiologic and ecologic investigations.

Intracellular Quantitation of Lactate Dehydrogenase
in Colorless Euglena gracilis and Astasia longa

Both soluble and particulate lactate dehydrogenase (LDH) have been described for Euglena gracilis Strain Z (22) and for Astasia longa Strain J (5). Reports of biochemical studies comparing the quantitative aspects of LDH from colorless E. gracilis and A. longa have not been found. Studies on the cytochemical demonstration of LDH in these species are also lacking.

A recent study by Berezina (4) has shown on a comparative basis, different levels of LDH and LDH isoenzymes in several protozoan species. When the procedure for the cytochemical demonstration of LDH (21) was applied to colorless E. gracilis and A. longa, different levels of cytochemical activity of LDH were observed in each species. Because of the structural similarity of the two species, and since the cells incubated in the presence of substrate and coenzyme exhibited qualitative differences in the amount of cytochemical reaction product, these organisms presented a suitable system for the intracellular quantitation of the reaction product of LDH in individual cells by rapid-scanning microspectrophotometry.

Materials and Methods:

Cultures of Euglena gracilis var. bacillaris Gross (colorless) and Astasia longa Pringsheim were obtained from the Indiana



Culture Collection (22) in Polytomella medium. The organisms were sub-cultured and maintained in Polytomella medium at room temperature (23-25 C) with continuous lighting.

Specimens were collected from log-phase cultures, washed twice in 0.135 M phosphate buffer (pH 7.0) and spread on glass slides. After air-drying, the incubation medium (21) consisting of 0.5 ml of 0.5 M Na-lactate, 0.3 ml of 0.1 M Tris buffer (pH 8.2), 0.17 ml of nitro-blue tetrazolium (NBT)(1 mg/ml), and 0.01 ml of 0.1 M nicotinamide adenine nucleotide (NAD) was added to the specimen slides and incubated at 37 C for 15 min (both the incubation medium and the specimen slides were pre-warmed to 37 C before adding the medium to the specimen). The 15 min incubation period was selected as optimum after comparing a series of specimens incubated for 10, 15, 20, 30 and 45 min at 37 C. Controls were prepared by incubation of specimens in a reaction mixture without Na-lactate or NAD. After the 15 min incubation period, the cells were placed in 10 per cent buffered formalin for 10 min, rinsed in 0.1 M Tris buffer, air-dried and mounted in glycerin (nD-1.455 at 20 C).

The absorption maxima of 550 mμ for the LDH cytochemical reaction product was determined by performing a wavelength absorption scan on random picked cells using the Universal Microspectrophotometer (UMSP-I). Control and incubated cells were scanned at 550 mμ with a scanning spot of 1.0 micron at 1.0 micron intervals throughout the cell using the Zeiss Scanning

Microscope Photometer (SMP) which was operated on-line with a PDP-12 computer. Cell scan data was collected on LINC tapes and later submitted to analysis by TICAS (31) programs using the PDP-12 computer.

Results:

Control preparations of both Euglena and Astasia showed no evidence of the presence of a reaction product when examined by light microscopy. Cells of Astasia incubated in the medium containing substrate (Na-lactate) and coenzyme (NAD) exhibited a distinct, but low level of the blue reaction product within the cell. On a qualitative basis, the distribution of the reaction product was rather uniform in the cell population. Incubated specimens of Euglena exhibited a higher level of activity as evidenced by an abundance of the reaction product in the cells.

Figure 24 is the digital matrix of a representative control cell of Astasia showing the non-specific absorption values (OD) of a cell scanned at 1.0 micron intervals at 550 mμ. Similar raw data images were obtained for the Euglena control cells. The points within the matrix where digits are missing represent areas of non-absorption. Such a phenomenon was apparently caused by the birefringence of a paramylum grain situated out of the focal plane, but lying in the measuring-beam light path. This phenomenon has previously been observed in these species when alcohol fixed, non-stained cells were examined at 260 mμ

(27). Examination of the digital matrix of all control cells scanned in this study revealed a similar random distribution of non-absorption points in each cell matrix. Figure 25 is an example of the digital matrix of an incubated Astasia showing the rather uniform intracellular distribution of the enzyme reaction product. Although many of the Euglena cells were characterized by a similar uniform intracellular dispersion of the reaction product which was present in a greater quantity, a number of cells exhibited uneven intracellular apportionment.

Figure 26 shows a histogram of the frequency of occurrence of the mean absorption in relative units (total absorption divided by the number of 1 micron spots measured throughout the cell) for control and incubated specimens of both species. The controls of both species were characterized by similar arrays of non-specific absorption, with a mean of 4 relative units for Astasia and 6 for Euglena. It should be noted, that if the number of non-absorption points within the digital matrix had been used in calculating the mean for each cell, the decrease of the mean for each control population would not have exceeded 1 unit (ie., the actual mean values for Astasia and Euglena would have been 3 and 5 respectively).

Qualitatively, when incubated Astasia cells were compared with incubated Euglena, it was estimated that the amount of enzyme reduction product in Euglena was 2-4 times that present

in Astasia. When the cell-scan data from both populations were compared (Fig. 26), the mean of the Euglena population was 3 times that of Astasia.

Discussion:

As far as it can be ascertained, this is the first report of the intracellular cytochemical quantitation of an enzyme reaction product in protozoans by microspectrophotometry. In contrast to microspectrophotometric procedures using the plug or core technique, where only one or a few measurements are obtained from a cell, the high-speed scanning stage of the SMP facilitates a rapid and uniform pattern of scanning and measurement recording of intracellular substance at a selected wavelength.

Qualitatively, one could only estimate if one or more dense, localized areas of the reaction product in some of the cells of Euglena would represent the same amount of reaction product in another cell which appeared to be less densely stained, but with an even intracellular apportionment. Since the entire cell could be scanned at 1.0 micron intervals, uneven intracellular localization of the reaction product, which was exhibited by some of the cells, did not present a problem in terms of quantitation.

The qualitative estimation of a relatively uniform distribution of the cytochemical reaction product from cell to cell in

the Astasia population was subsequently shown to have a variation in mean of 10-24 relative units per cell (Fig. 26). Euglena, however, was characterized by both even and uneven distributions of the LDH reaction product within the cell, with a mean ranging from 30-64 relative units per cell. Although there was some evidence of a size-quantity relationship, further studies would have to be initiated to determine if a correlation could be made between quantity of enzyme reaction product and cell cycle stage.

In addition to finding distinct qualitative differences in the cytochemical reaction product of LDH when comparing these two species, this study has also shown that these differences could be quantitated on an individual cell basis. At the same time, it is realized that the properties of these organisms depend largely upon the conditions employed in culturing, and that LDH levels, as well as other enzymes, are likely to vary when cultured in different media and with different light and temperature conditions.

If a conventional assessment on a qualitative basis had been made of the control and incubated cells, the scoring would have been:

	<u>Astasia</u>	<u>Euglena</u>
Control	-	-
Incubated	+	++, +++

Although this does provide a comparative estimate of the LDH in

the two species, the determinations are arbitrary. From the data accumulated by rapid-scanning microspectrophotometry, it has been shown that these procedures could be effectively applied to establishing a quantitative comparison of the LDH reaction product of the two species.

From this study it has been shown that cytophotometry of individual cells can lead to useful results in quantitation of cytochemical reaction products within cells. There will continue to be various problems encountered because of enzyme specificity, competition, inhibition and other factors affecting enzymes, however, this approach is a significant step toward quantitation as compared with earlier methods, which at best, were only qualitative.

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Explanation of Figures

- Fig. 1. An outline for data acquisition and analysis using a rapid scanning microspectrophotometer and a computer.
- Fig. 2-3. An outline of the procedure used for lymphocyte separation from a peripheral blood sample and subsequent specimen slide preparation of microspectrophotometric analysis.
- Fig. 4. Raw data matrix of a G-1 lymphocyte. The cell was scanned with a 0.5 micron measuring spot at 0.5 micron at 260 mμ. The isobar lines were arbitrarily placed within the matrix to illustrate the intracellular pattern change as changes occur relative to nucleoprotein during the cell cycle.
- Fig. 5. Raw data matrix of a G-1 lymphocyte.
- Fig. 6. Raw data matrix of a lymphocyte in the early S phase.
- Fig. 7. Raw data matrix of a lymphocyte in S phase.
- Fig. 8. Raw data matrix of a lymphocyte where S phase has been completed and chromatin condensation has begun.
- Fig. 9. Raw data matrix of a lymphocyte in the metaphase stage of the cell cycle.
- Fig. 10. Raw data matrix of a dividing (M or mitotic) lymphocyte.
- Fig. 11. Raw data matrix of a pair of daughter cells (G-2) which have not completed cytoplasmic separation, however, note that decondensation of the chromatin has occurred.
- Fig. 12. A table of microspectrophotometric data showing the distribution of several parameters for a series of cells

showing the complete cell cycle. This data was collected from specimens which were alcohol fixed and unstained.

Fig. 13-14. Another series of cells analyzed by microspectrophotometry showing the distribution of OD values in Papanicolaou stained lymphocytes and scanned at 530 nm.

Fig. 15. A population histogram showing the distribution of the frequency of occurrence of the cumulative OD values from control and 45-50 per cent infected tissue culture cells inoculated with Coxsackie B-5 virus.

Fig. 16. A population histogram showing the distribution of the frequency of occurrence of the cumulative OD values from control and 95 per cent infected tissue culture cells inoculated with Coxsackies B-5 virus. The controls from this population show almost identical overlap with the controls of Fig. 15 even though the specimens were prepared 48 hrs apart.

Fig. 17. A simplified diagram showing how data can be edited to remove undesirable portions of data from the cell-scan area which does not contribute to the cell matrix which is of primary interest. Many times the cell selected for scanning is adjacent to another cell or to debris. It is much easier to scan the excess material, then remove the unnecessary data points using the computer and a routine specifically designed to perform this task.

Fig. 18. Using the editing routine, equiprobable distribution profiles of cells were prepared from the cumulative population data. Once the data value parameters were obtained for the high 25 per cent and the low 25 per cent of the OD values from a population, then each cell was processed and a cytoplasmic (low 25 percent) and nuclear (high 25 per cent) profile was obtained.

Fig. 19. This figure shows the maximum and minimum profiles from the control populations and the infected populations. Each population was characterized by falling within the limits shown. When the parameters for the control cells were applied to the cells of the infected population, each cell could be designated as similar (an uninfected cell) or dissimilar (an infected cell). When the population survey was completed, the percentage of infected cells was in close correlation with what had originally been estimated on the basis of cytopathic effect factors.

Fig. 20. Raw data matrix of Euglena gracilis scanned at 1 micron intervals at 260 mμ. The nucleus of the cell is outlined within the matrix.

Fig. 21. Population histogram showing the distribution of total OD value for each cell of both populations. Over 100 cells in each population was scanned. On the basis of total OD, a separation of species was not possible.

- Fig. 22. When data values from cell nuclei were compared, it was apparent that the Pattern Search routine would likely result in a high degree of separation for the 2 species. This figure shows the frequency of occurrence of a specific nuclear pattern applied to each cell of both populations.
- Fig. 23. An example of the specific nuclear pattern with 4 "fits" as applied to the raw data matrix for Euglena.
- Fig. 24. Raw data matrix of Astasia control specimen showing the non-specific absorption at 550 mμ.
- Fig. 25. Raw data matrix of an incubated Astasia cell showing the distribution of LDH reaction product with the cell.
- Fig. 26. A graph showing the distribution of cumulative average OD (average extinction) for the cells of each population. On the basis of the amount of cytochemical LDH reaction product within the cells, a distinct quantitative difference was shown.

DATA ACQUISITION AND ANALYSIS

PERCENT TRANSMISSION OF INDIVIDUAL
POINTS THROUGHOUT THE SPECIMEN

↓
PHOTOELECTRIC CELL

↓
ANALOG-TO-DIGITAL CONVERTER → HIGH SPEED PAPER TAPE PUNCH

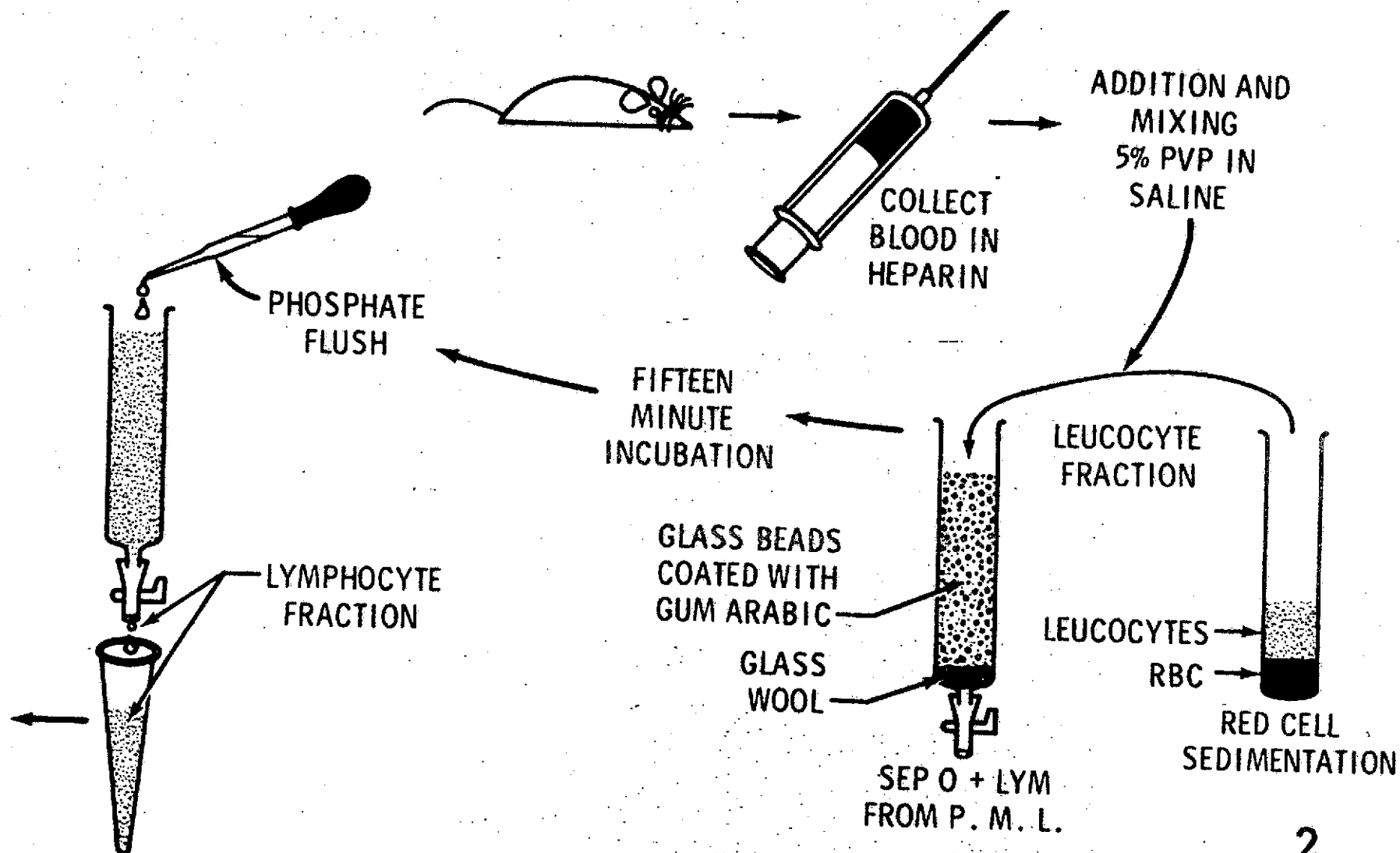
↓
PDP-12 COMPUTER ←

↓
RAW DATA TAPE → RAW DATA PRINT-OUT OF CELL

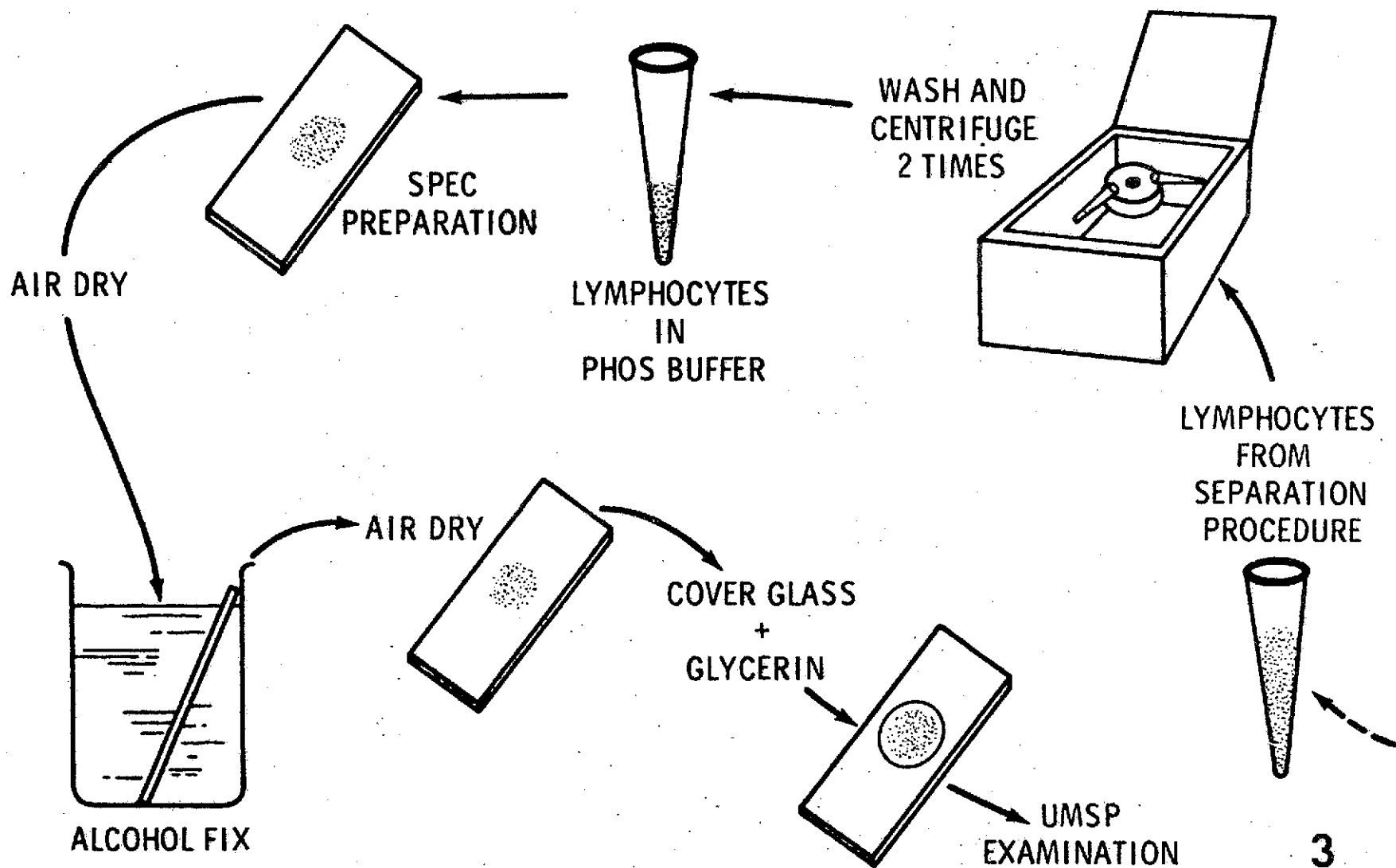
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REDUCED DATA TAPE

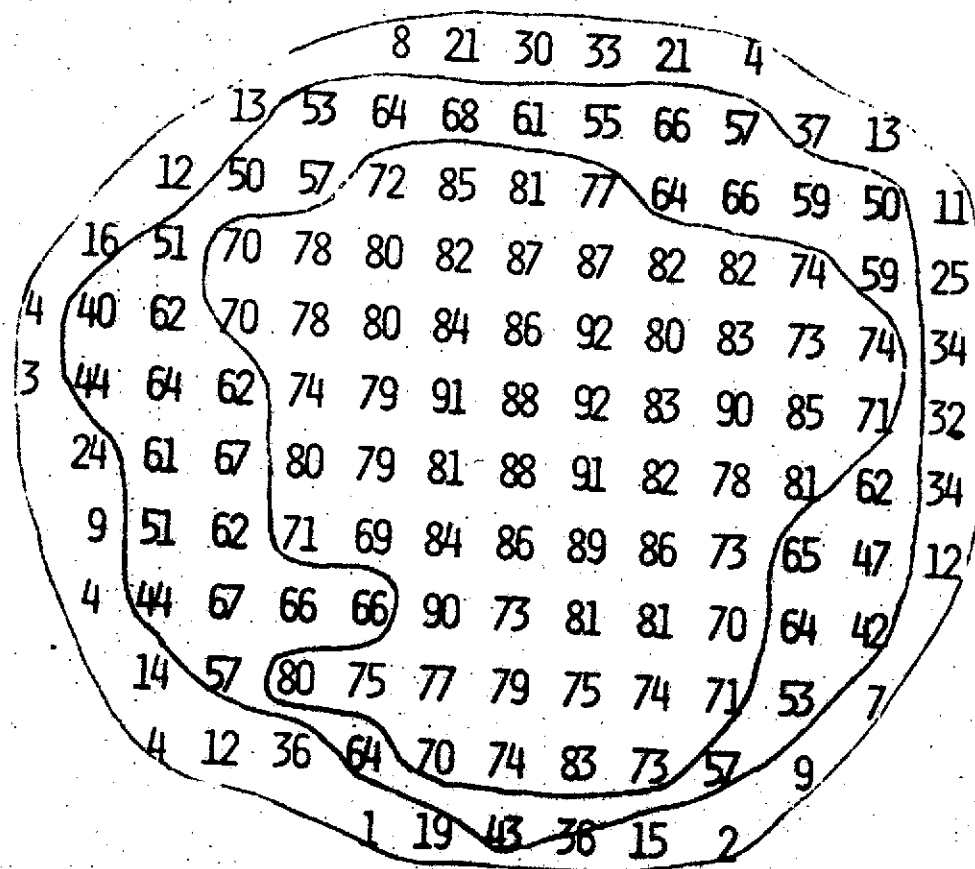
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APPLICATION OF COMPUTER
PROGRAMS FOR DATA
ANALYSIS

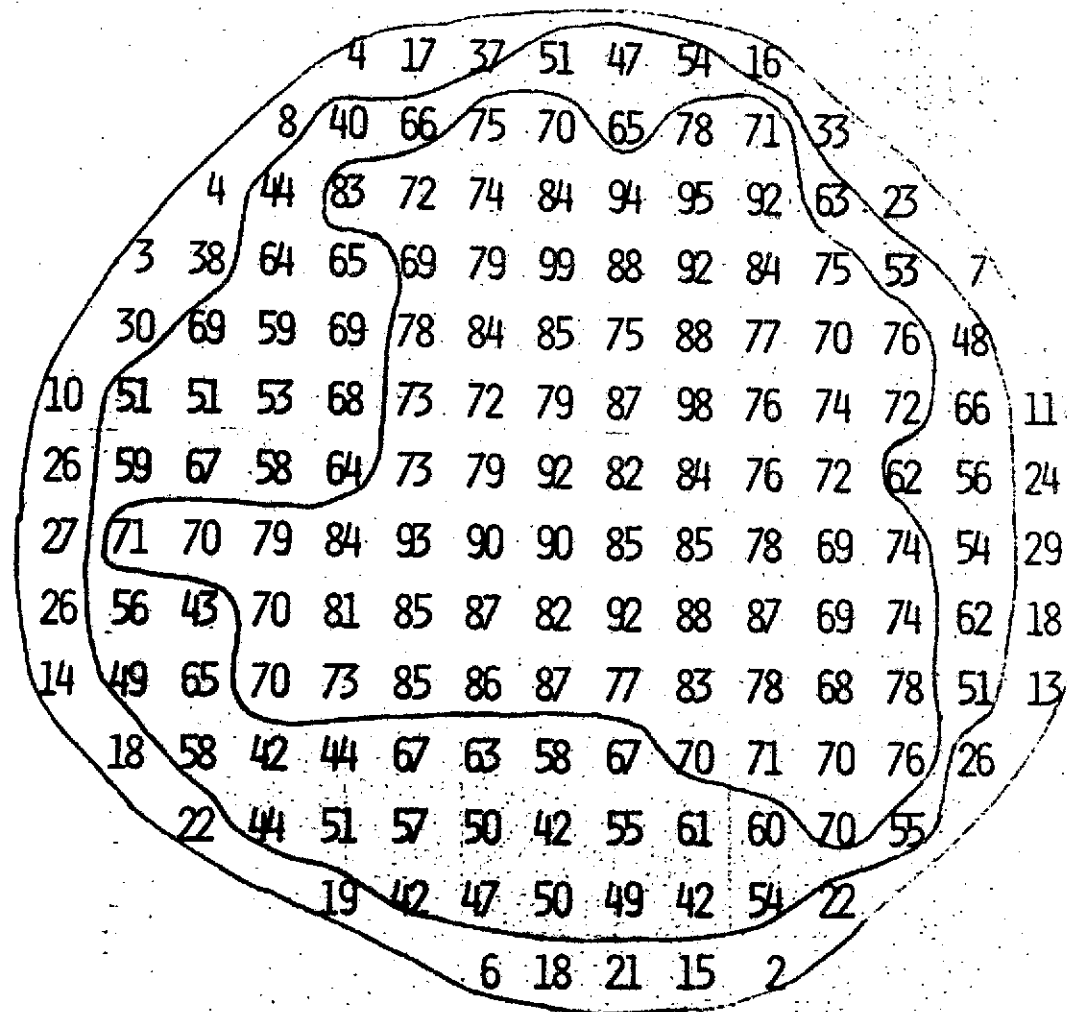
LYMPHOCYTE SEPARATION PROCEDURE

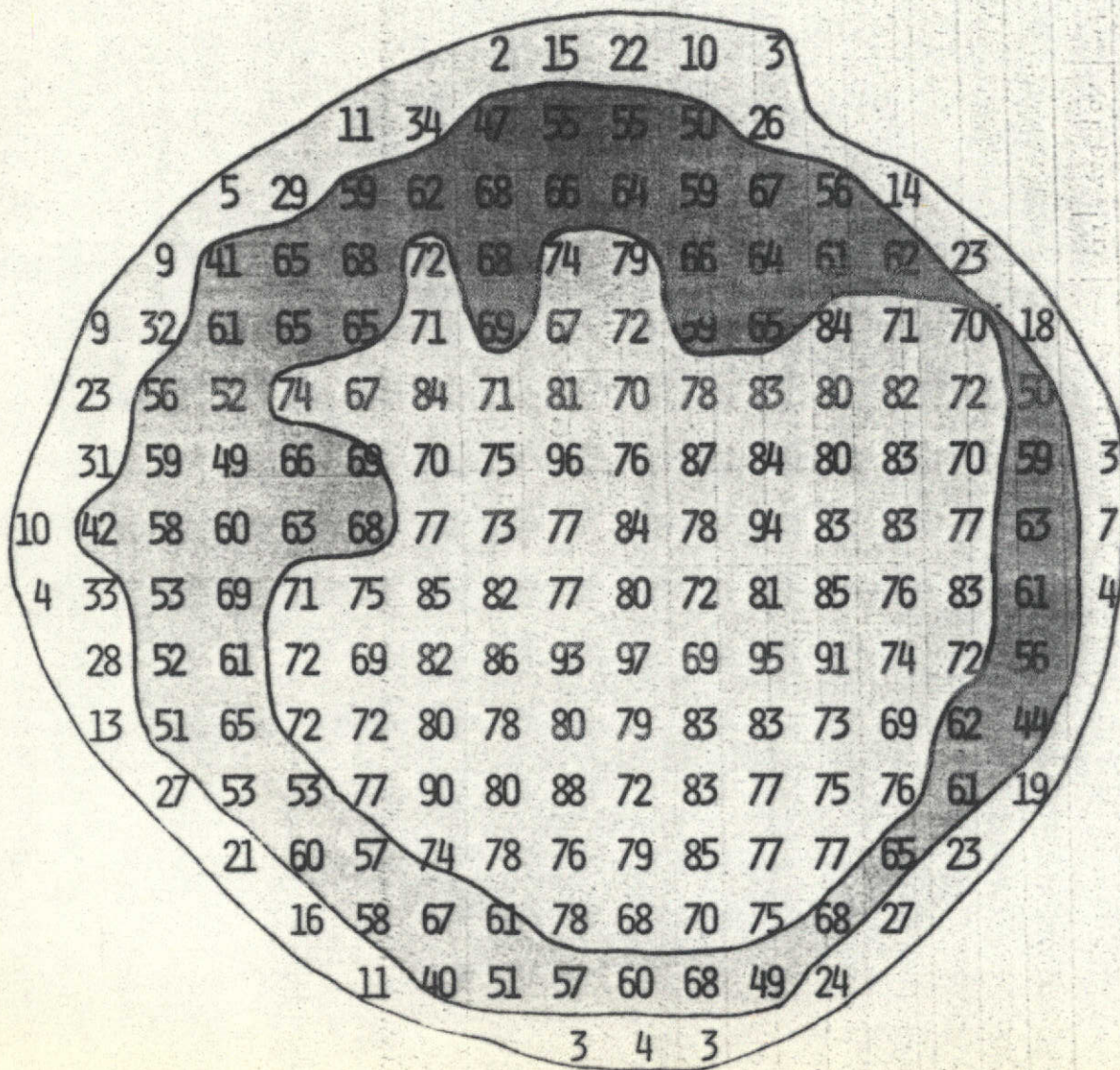


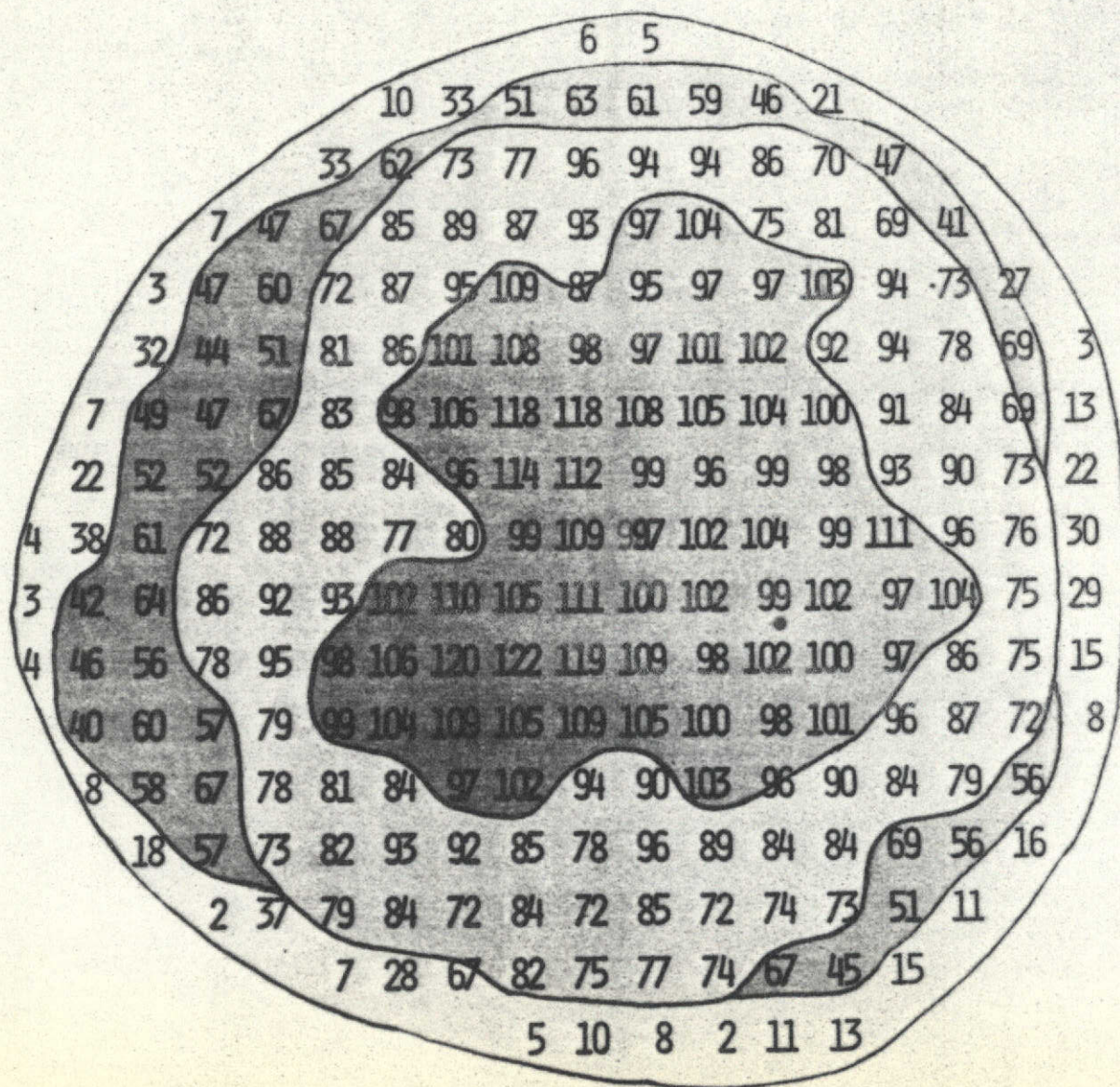
SPECIMEN SLIDE PREPARATION

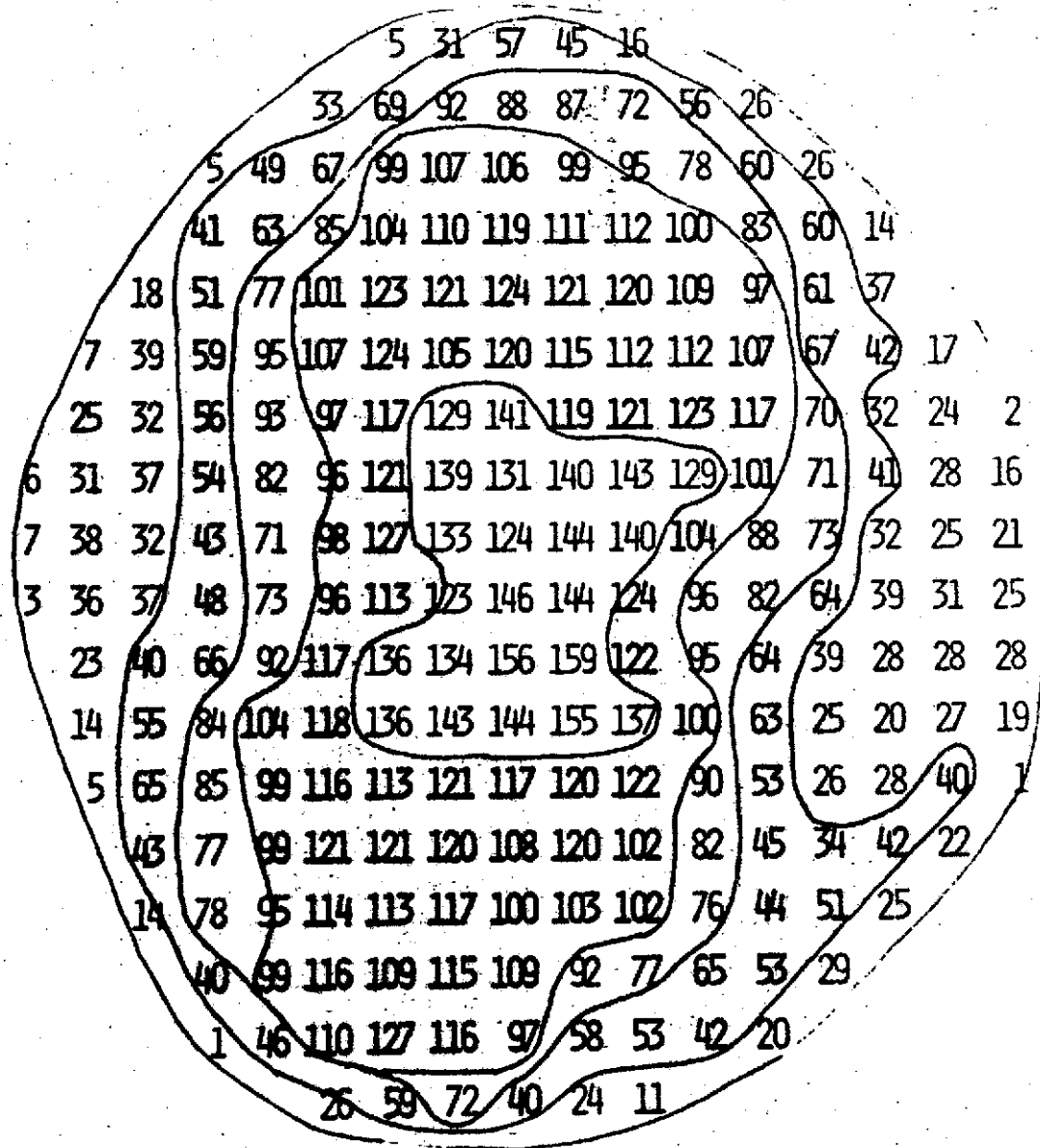


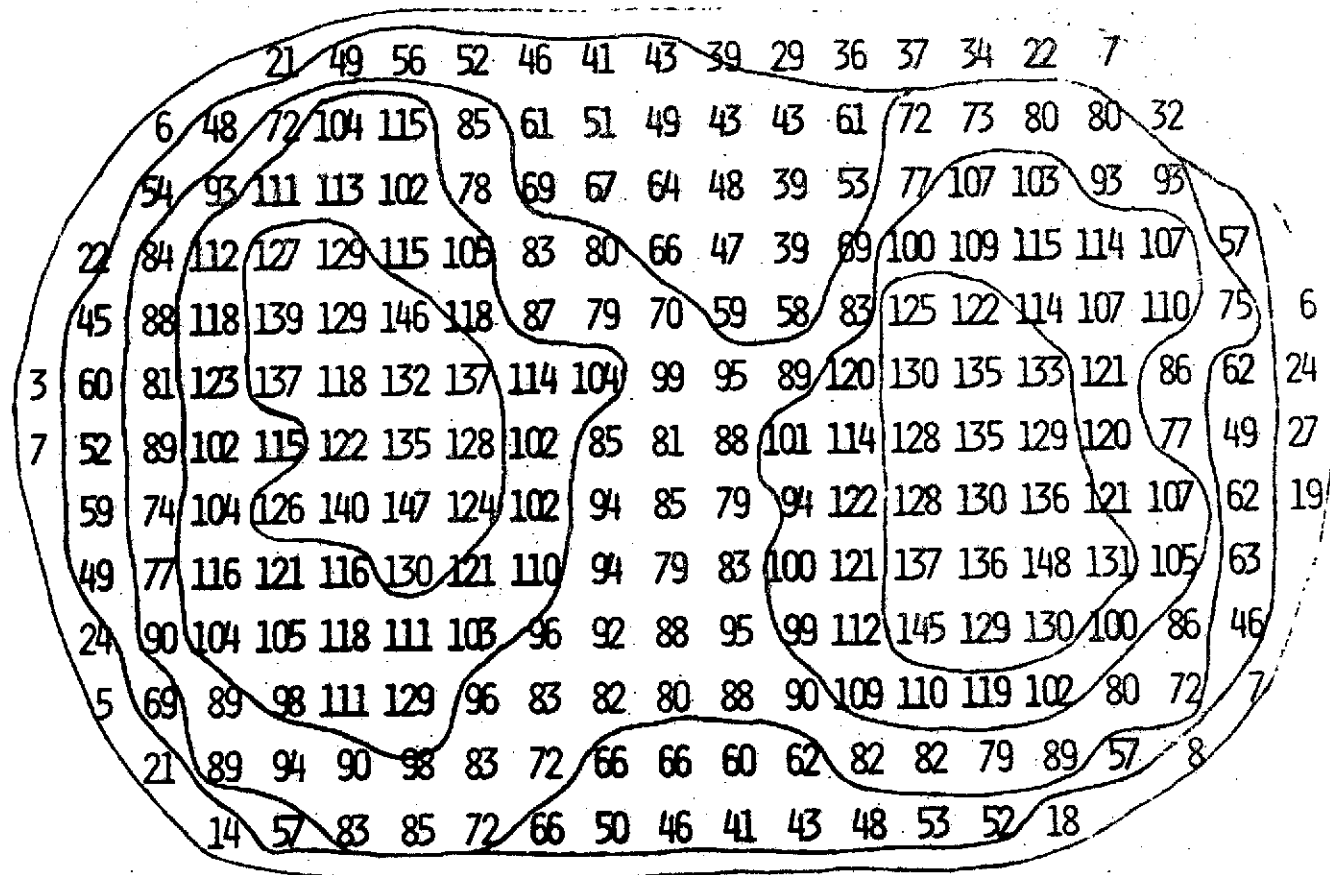


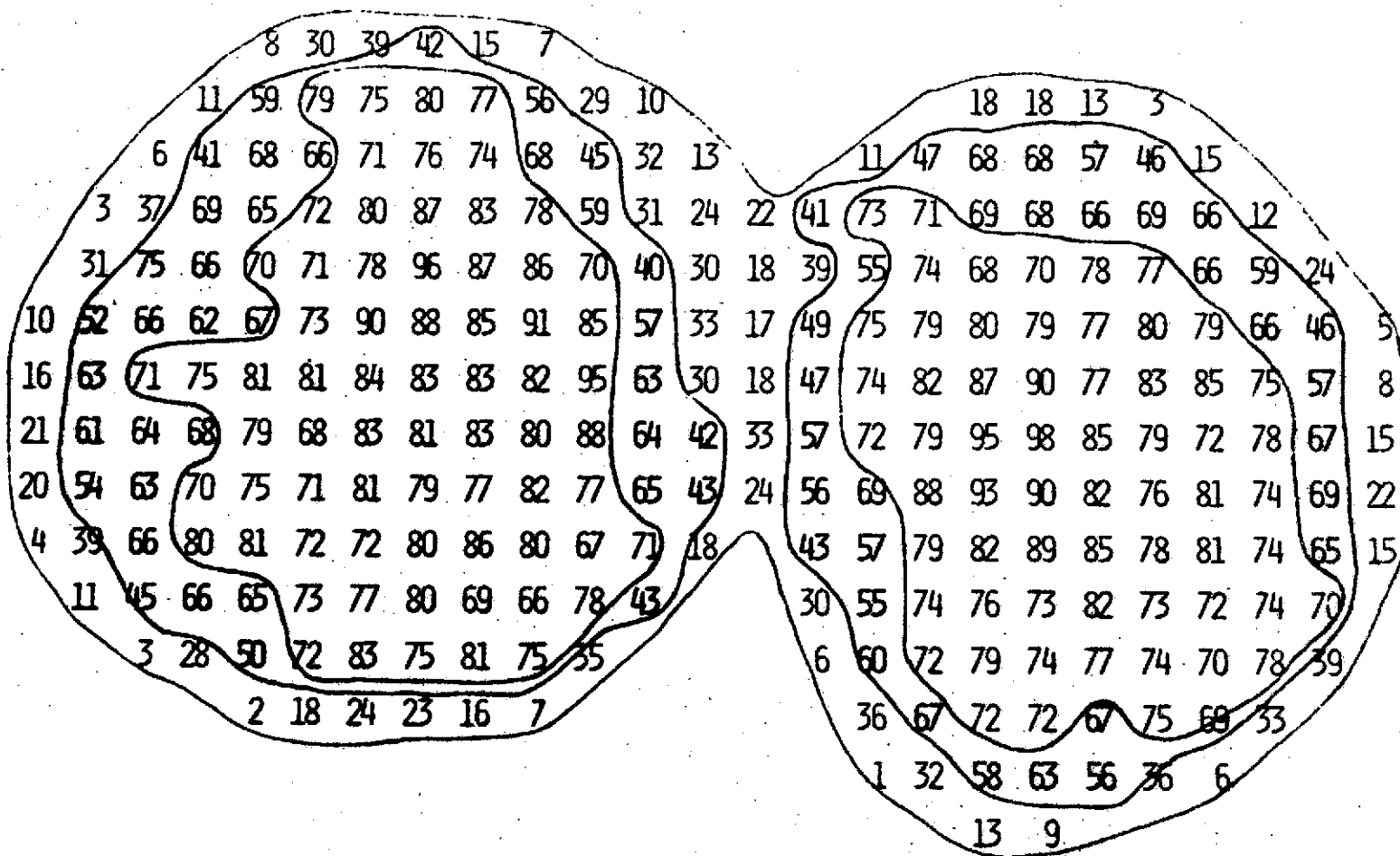












CELL DIVISION - UNSTAINED

CELL NO.	TOTAL EXTINCTION AT 260 MU	RELATIVE AREA (NO. 0.5U SPOTS)	AV. EXTINCTION	SUM OF 5 HIGHEST NOS.
1	65	148	.44	3.40
2	81	191	.42	3.34
3	100	178	.56	5.17
4	138	210	.66	5.80
5	164	293	.56	6.79
6	141	219	.64	5.72
7	142	258	.55	5.61
8	151	343	.44	3.83
—	—	—	—	—
8A	70	179	.39	3.39
8B	81	181	.45	3.83

CELL DIVISION - PAPANICOLAOU STAIN

<u>CELL NO.</u>	<u>TOTAL EXTINCTION AT 590 MU</u>	<u>RELATIVE AREA (NO. 0.5 U SPOT)</u>	<u>AV EXTINCTION</u>	<u>SUM OF 5 HIGHEST NOS.</u>
1	77	132	.59	4.56
2	97	160	.61	4.78
3	116	195	.59	4.75
4	136	205	.66	5.04
5	167	227	.73	5.97
6	174	244	.71	6.99
7	179	230	.73	7.60
8	201	240	.83	7.26
9	145	320	.45	4.37
10	157	267	.59	4.72

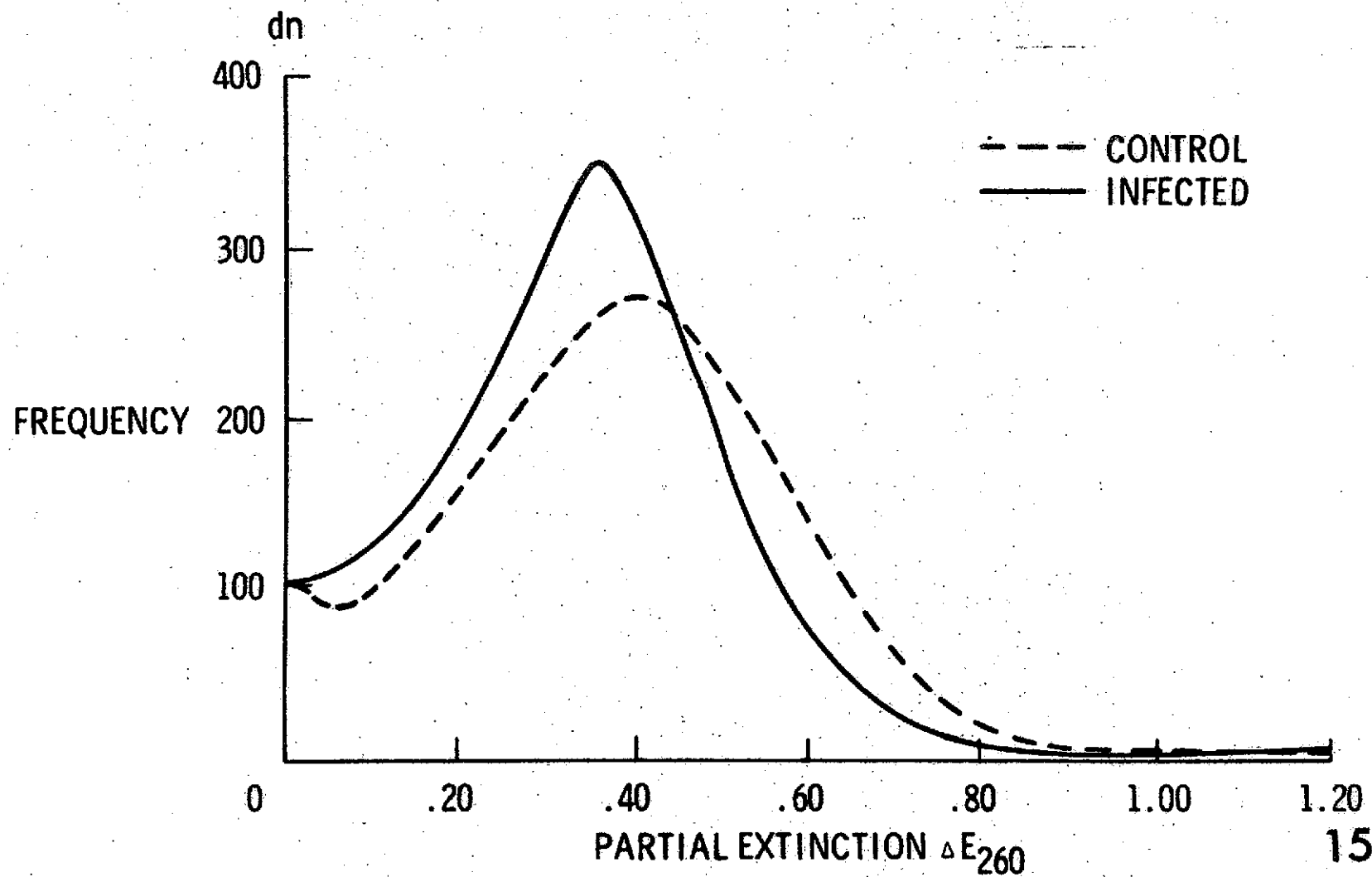
CELL DIVISION - PAPANICOLAOU STAIN (CONT)

COMPARISON OF JUVENILE CELL (NO. 1) WITH UNSEPARATED DAUGHTER CELLS (NO. 9-10)

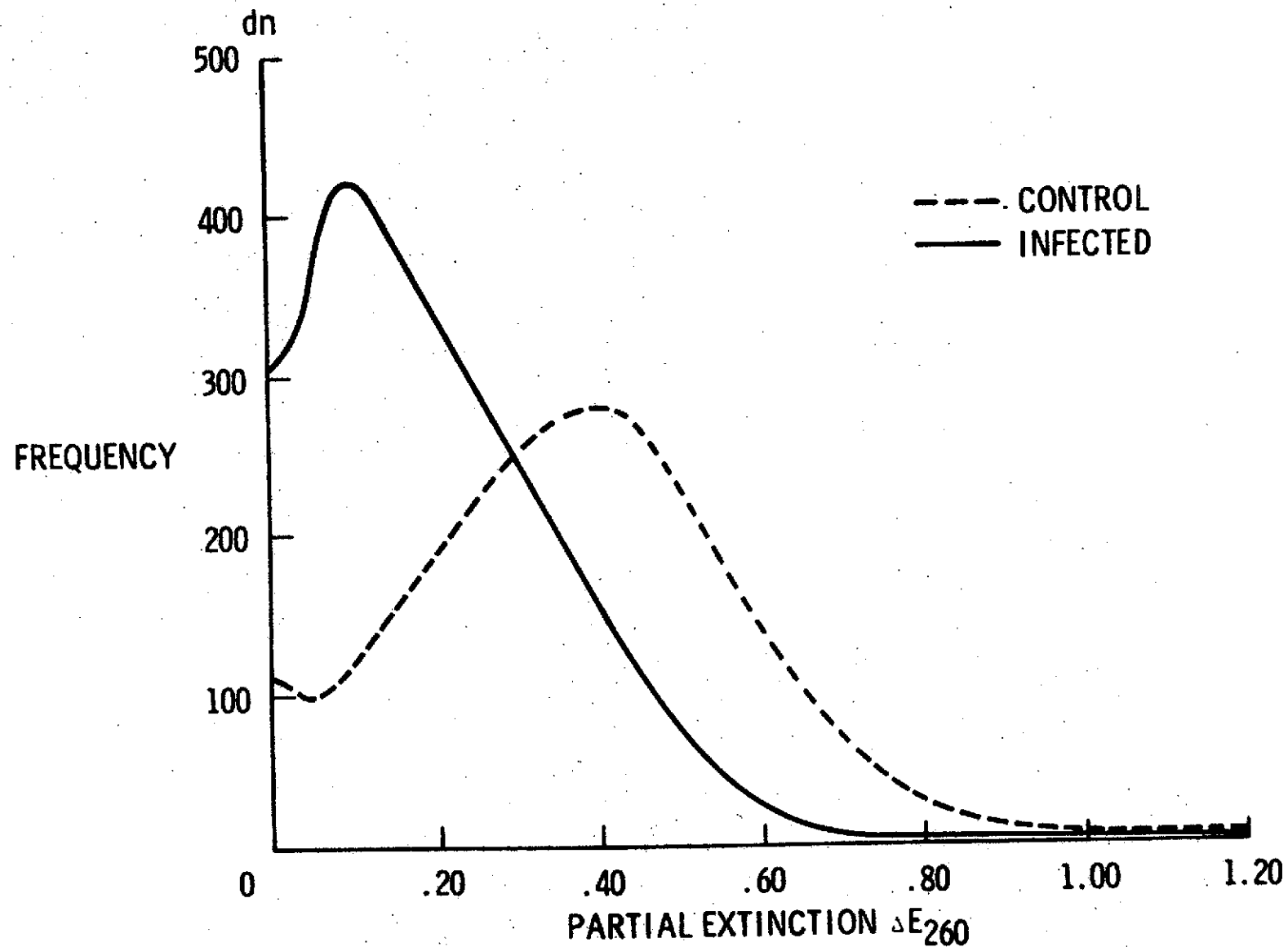
<u>CELL NO.</u>	<u>TOTAL EXTINCTION AT 590 MU</u>	<u>RELATIVE AREA (NO. 0.5 U SPOTS)</u>	<u>AV EXTINCTION</u>	<u>SUM OF 5 HIGHEST NOS.</u>
1	77	132	.59	4.56
9a	69	159	.43	4.03
9b	75	161	.47	4.37
10a	82	139	.59	4.60
10b	75	128	.58	4.66

POPULATION HISTOGRAM - GROUP I

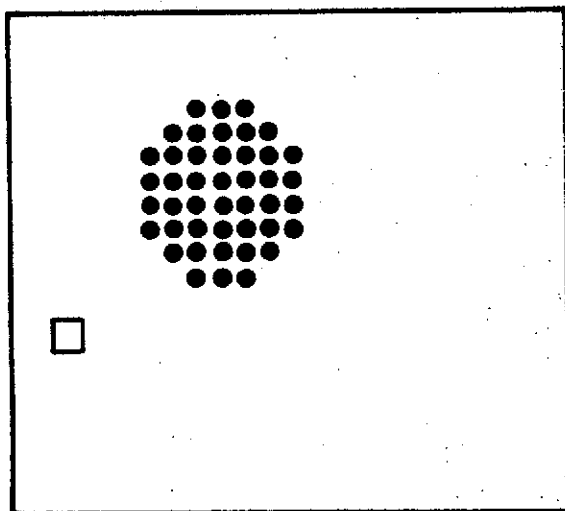
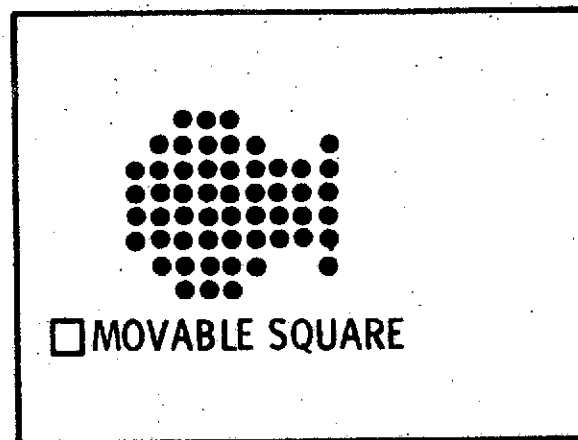
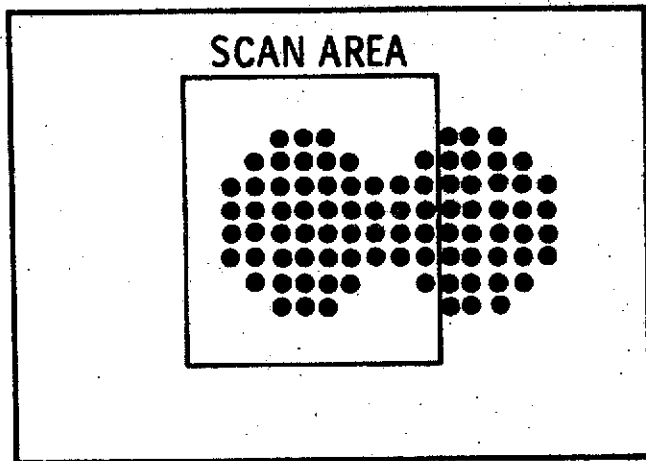
AM - GROU



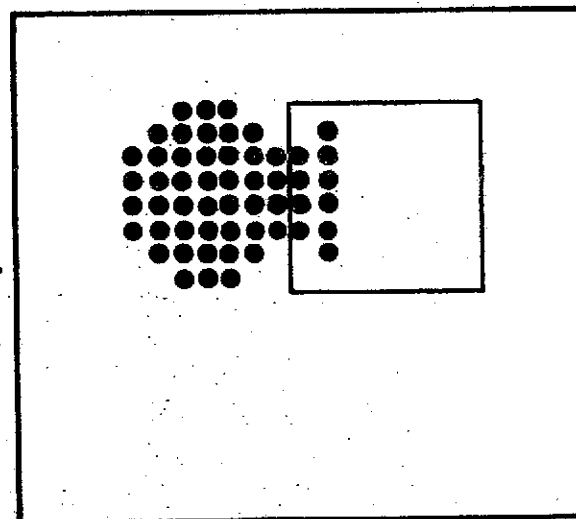
POPULATION HISTOGRAM - GROUP II



CELL VIEW AND DELETION PROGRAM

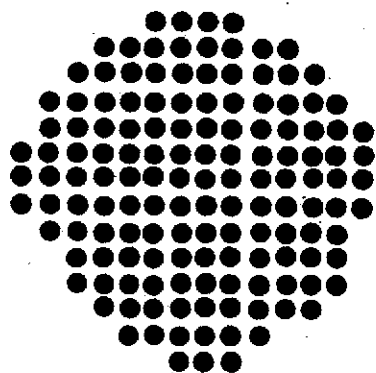


CLEANED CELL

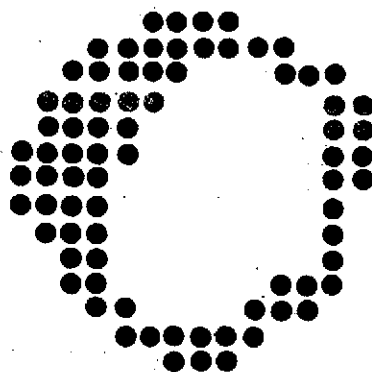


SHIFT ALL POINTS INSIDE SQUARE

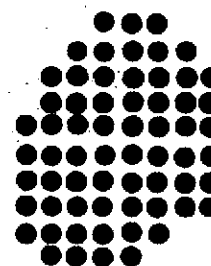
CELL VIEW AND DELETION PROGRAM (CONT)



DISPLAY OF WHOLE CELL

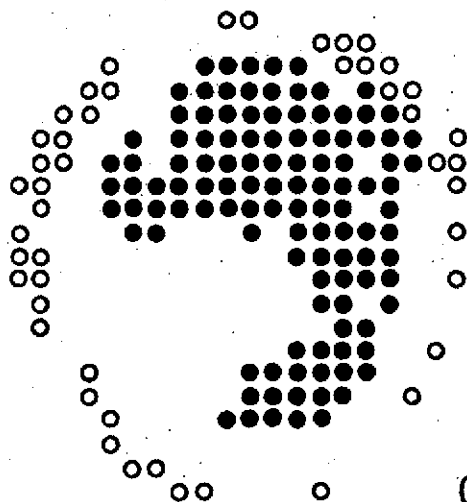


DISPLAY OF VALUES
BETWEEN 0.01 - 0.35

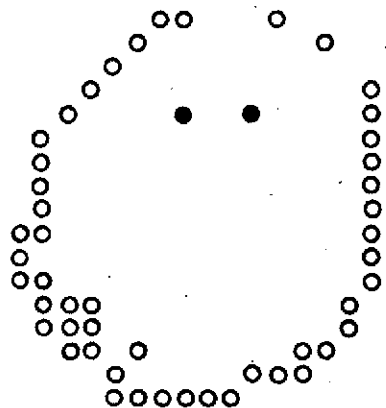
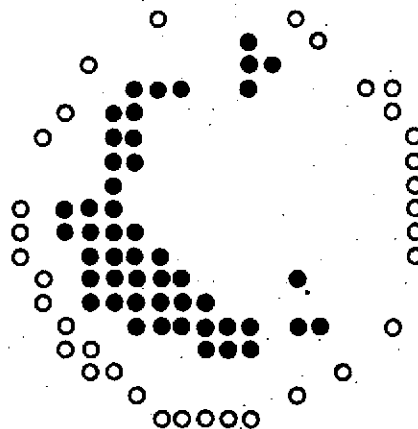


DISPLAY OF VALUES
BETWEEN 0.36 - 1.00

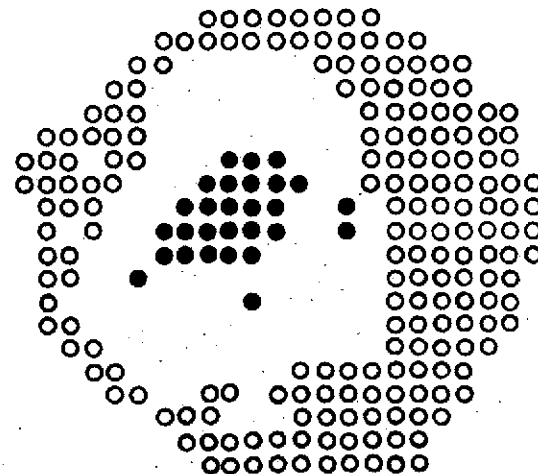
EQUIPROBABLE DISTRIBUTION PROFILE

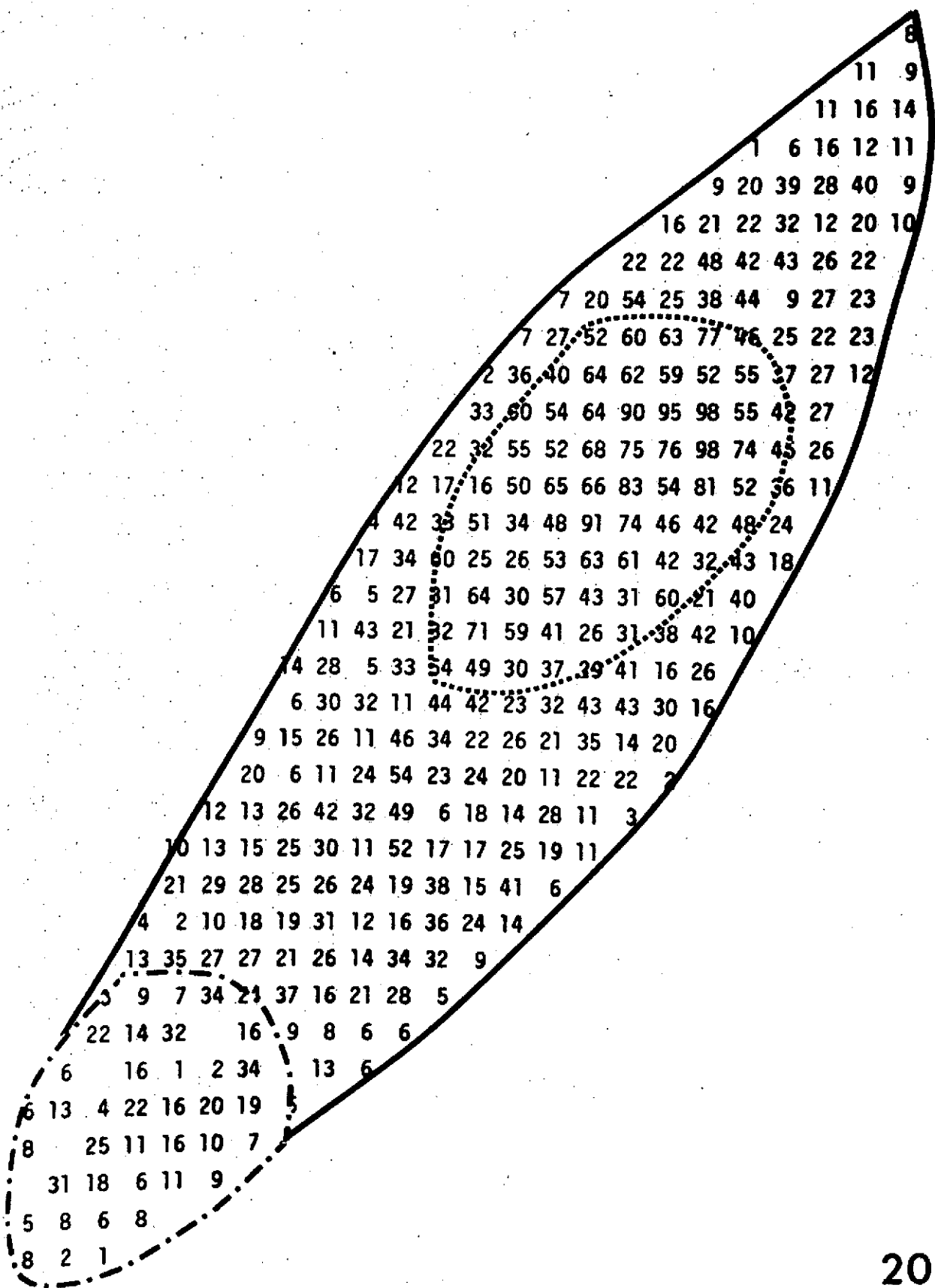


CONTROL

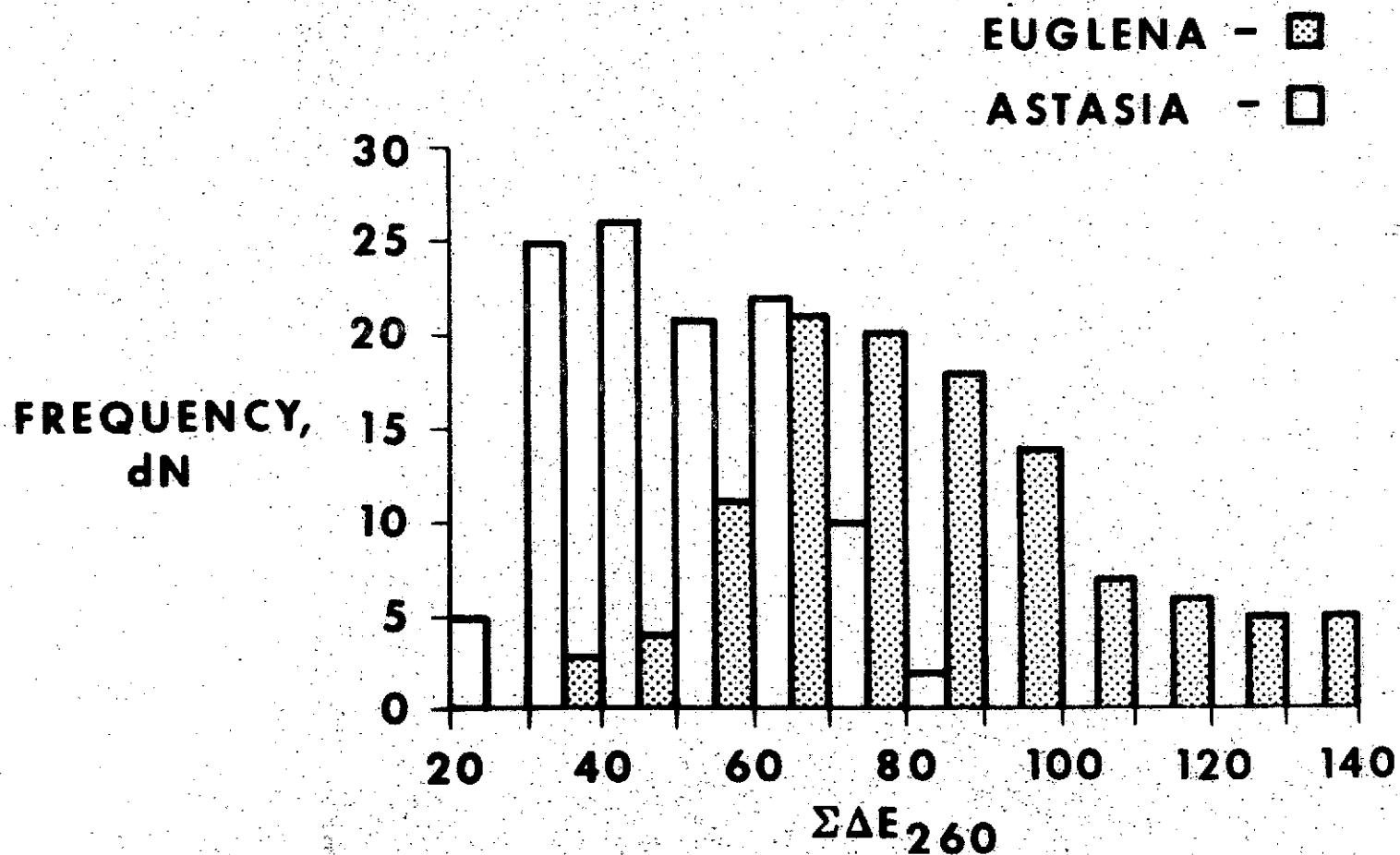


INFECTED

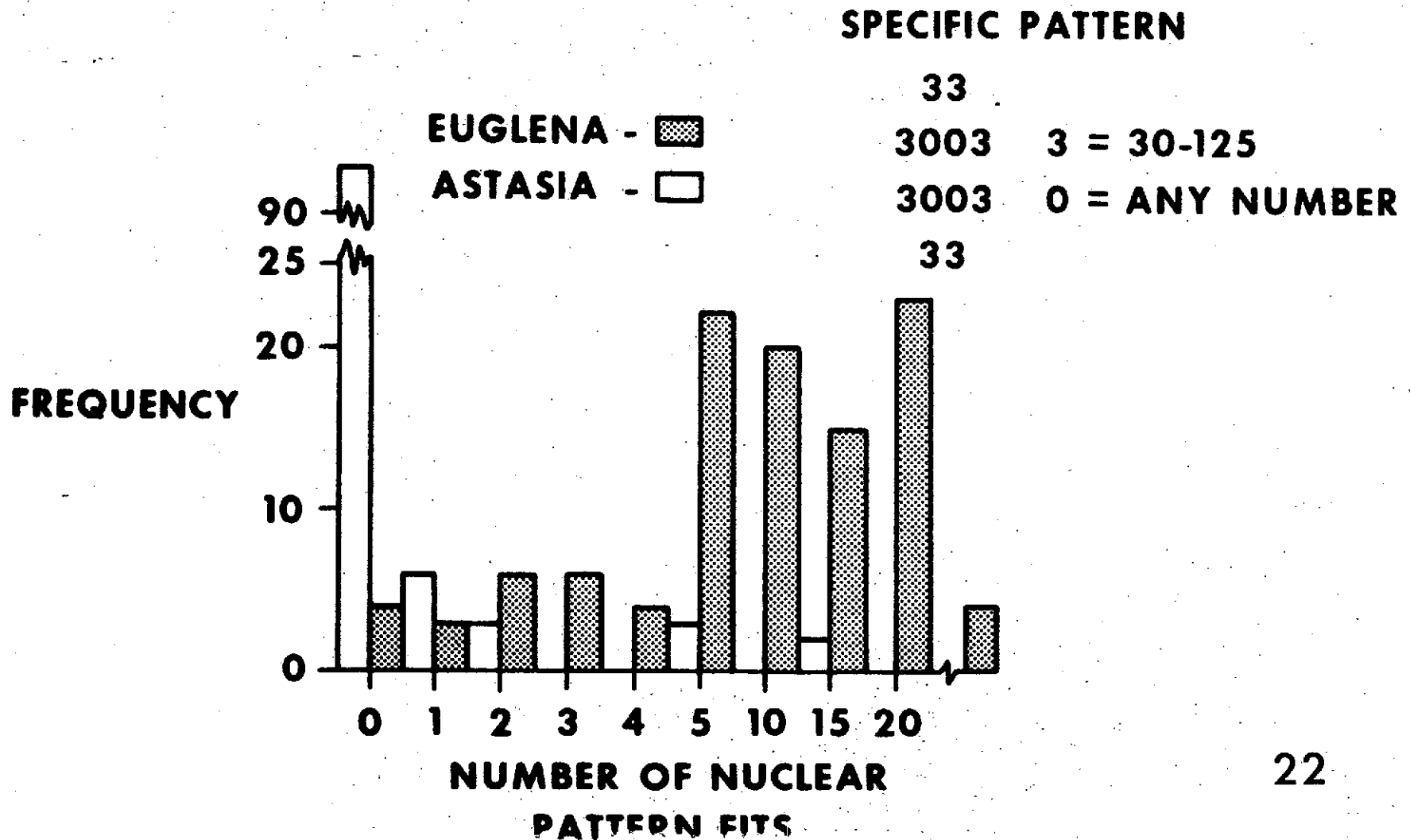




EUGLENA GRACILIS (COLORLESS) AND ASTASIA LONGA



SPECIFIC NUCLEAR PATTERN FOR EUGLENA AND ASTASIA



NUCLEAR PATTERN FIT FOR EUGLENA

[illegible]

24

NASA-S-71-4051-S

LDH REACTION PRODUCT IN ASTASIA

1 2 2
6 21 18 13 2
18 30 13 20 7 6
1 21 32 15 14 14 22 9
1 32 58 31 22 23 37 24 12
22 29 29 33 36 51 35 34 11
20 59 27 45 41 41 46 44 34 9
1 11 28 21 23 45 38 46 26 25 28 10
17 42 27 18 34 28 49 36 34 40 34 9
12 27 20 41 35 36 20 22 36 14 24 36 6
10 43 35 23 34 21 26 42 29 36 22 38 27 5
5 37 31 45 29 17 16 33 17 24 34 31 27 15 7
1 20 21 24 30 22 11 26 28 37 33 29 22 14 25 8
3 22 27 26 14 25 27 10 20 12 34 26 25 29 25 6
1 9 29 28 30 36 27 26 29 27 36 24 11 29 23 19 6
3 20 26 21 40 22 24 18 25 32 24 13 22 47 19 19 7
7 27 27 26 28 29 32 22 23 44 15 8 18 23 21 20 5
2 13 32 19 28 24 26 33 22 33 37 19 23 18 18 22 18 3
4 19 34 34 31 40 22 23 22 27 19 35 29 37 26 23 11 1
4 21 34 40 37 31 27 19 25 27 28 27 20 24 34 14 8
5 22 46 42 56 42 33 28 27 24 22 20 27 31 29 11 6
5 20 36 64 34 49 29 16 18 17 29 13 25 34 30 16 2
5 17 28 43 63 52 20 24 31 31 10 18 27 21 37 10
3 17 27 45 49 43 41 38 36 15 26 42 11 20 17 2
2 11 38 54 51 49 54 31 33 58 55 17 25 27 5
7 26 26 26 37 44 25 36 20 24 19 22 6
4 17 21 32 42 34 25 34 33 31 16 5
3 17 29 52 53 45 43 43 26 11 1
3 16 32 46 34 30 20 13 4
1 8 15 11 7 4 1
1 7 2

DISTRIBUTION OF LDH REACTION PRODUCT IN ASTASIA AND EUGLENA

